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Assessment of Chemical Composition of High Molecular Fraction from Medicinal Plant *Onosma sericea* Willd. by Fourier Transform Infrared Spectroscopy and Nuclear Magnetic Resonance Technique

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ABSTRACT

Background: In our previous studies we investigated water-soluble mucilaginous high molecular fractions (HMF) of medicinal plants *Symphytum asperum*, *S.caucasicum*, *S.grandiflorum*, *S.officinale*, *Anchusa italica*, *Cynoglossum officinale*, *Borago officinalis* and *Paracynoglossum imeretinum* (Boraginaceae family). The water extracts of aforenamed plants were fractionated by ultrafiltration on membrane filters with cut values of 1000 kDa or 500 kDa. This fractionation procedure allowed us to remove most polysaccharides and to obtain water-soluble HMFs. The main chemical constituent of HMFs of plants described above was found to be biologically active unique caffeic acid derived biomacromolecule poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] that is poly[3-(3,4-dihydroxyphenyl)glyceric acid].

Objectives: Within our ongoing search for biologically active biopolymers in plant species belonging to different genera of the Boraginaceae family, the present study aimed to isolate and investigate a water-soluble high-molecular mucilage fraction (Mr>500 kDa) of *Onosma sericea* roots (HMF-OR) in order to study main chemical constituents of HMF-OR and carry out their structure elucidation.

Methods: HMF-OR was isolated from water mucilage extract of *O.sericea* roots utilizing ultrafiltration with a membrane filter with a cut-off value of 500 kDa, as described in some of earlier publications. Analyses of HMF-OR were carried out by nondestructive physicochemical instrumental methods Fourier Transform Infrared Spectroscopy (FTIR), which is considered one of the most effective methods to identify the functional groups and Nuclear Magnetic Resonance (NMR) techniques which is a powerful tool for structure elucidation of chemical compounds.

Results: According to data of FTIR and NMR spectroscopies, one of the main chemical constituents of HMF-OR was found to be novel *p*-coumaric acid-derived biopolymer, namely poly[oxy-1-carboxy-2-(4-hydroxyphenyl)ethylene], that is poly[3-(4-hydroxyphenyl)glyceric acid] (PHPGA). Besides, the data also reveals in HMF the presence of complex pectin type polysaccharide.

Conclusions: PHPGA is a regular polymer with a residue of 3-(4-dihydroxyphenyl)glyceric acid as the repeating unit. The polyoxyethylene (polyethylene glycol) (PEG) chain is the backbone of PHPGA. 4-Hydroxyphenyl and carboxyl groups are regular substituents at two carbon atoms in the chain. The complex pectin type polysaccharide has consisted of a disaccharide repeating unit [→α-D-GalpA-1,2-α-L-Rhap-1,4→] backbone, with side chains contained highly branched α-(1→5)-linked arabinan and short linear β-(1→4)-linked galactan, attached to O-4 of the rhamnosyl residues.

Keywords: *Onosma sericea*; pectin; poly[3-(4-hydroxyphenyl)glyceric acid]; poly[oxy-1-carboxy-2-(4-hydroxyphenyl)ethylene]; polysaccharide.

INTRODUCTION

Biopolymers are naturally occurring polymers found in living organisms such as plants, animals, microbes, and other natural sources. The usage of biomaterials is becoming more popular as a means of reducing the use of nonrenewable resources and reducing environmental pollution produced by synthetic materials. Biopolymers' biodegradability and nontoxic nature help to maintain our environment clean and safe. Biopolymers' unique nontoxicity, biodegradability, biocompatibility, and ecofriendly features are boosting their applications, especially in bioengineering fields, including agriculture, pharmaceuticals, biomedical, ecological and etc.[1]

In our previous studies we investigated watersoluble mucilaginous high molecular fractions (HMF) of medicinal plants *Symphytum asperum*, *S.caucasicum*, *S.grandiflorum*, *S.officinale*, *Anchusa italica*, *Cynoglssum officinale*, *Borago officinalis* and *Paracynoglossum imeretinum* (Boraginaceae family). The water extracts of aforenamed plants were fractionated by ultrafiltration on membrane filters with cut of values of 1000 kDa or 500 kDa. This fractionation procedure allowed us to remove most polysaccharides and to obtain water-soluble HMFs. The main chemical constituent of HMFs of plants described above was found to be biologically active unique caffeic acid derived

biomacromolecule poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] that is poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDHPGA).[2-8] The structure elucidation of PDHPGA was carried out according to data of FTIR spectra and different techniques of NMR spectroscopy.[2-9] Unlike the above-mentioned plants, PDHPGA was not detected in the following species of Boraginaceae family: *Asperugo procumbens*, *Aegonichon purpurocaeruleum*, *Echium rubrum* and *Lythospermum officinale*.

PDHPGA exhibited a wide spectrum of biological activities due to the presence of numerous catechol groups covalently linked to the polyethylene glycol backbone of macromolecule. PDHPGA consequently showed immunomodulatory (anticomplementary), antioxidant, anti-inflammatory, wound and burn healing, anti-microbial, and anticancer properties.[2,3,9-14]

It is necessary to emphasize that HMFs of above listed plants, besides major component PDHPGA, contained some minor amount of residual complex polysaccharides, namely pectin type acidic arabinogalactan and/or rhamnogalacturonan.[2-8]

Onosma L. (Family: Boraginaceae) is one of the largest genera of the tribe Lithospermeae Dumort. In Boraginaceae, representing 200 species in Asia and Europe. Onosma species are traditionally valued as a remedy for rheumatism, bladder pain, kidney irritation and palpitation of heart, laxative, blood diseases, bronchitis, leukoderma, fever, wounds, burns, piles and urinary calculi. Phytochemicals, including lipids, pyrrolizidine alkaloids, phenolic compounds, naphthoquinones, and flavonoids are reported from different *Onosma* species. Some of the traditional uses of various species have been scientifically validated and are shown to possess antioxidant and anti-inflammatory, spasmolytic, antimicrobial, analgesic, antitumor, wound healing potential.[15]

METHODS

Apparatus

The Fourier's transformed infrared (FTIR) transmission spectrum was carried out in KBr disc through an FTIR spectrophotometer Jasco FT/IR-4600 (Made in Japan). One-dimensional ^{1}H and ^{13}C NMR spectra were recorded for 1% solutions in D_2O at 353°K with a Bruker Avance III 400 spectrometer (Uster, Switzerland), operating frequencies of 400.13 and 100.57 MHz, respectively. Acetone was used as internal standard ^{1}H ($C\underline{H}_3$) at δ_H 2.69 ppm and ^{13}C ($\underline{C}H_3$) at δ_C 31.45 ppm relative to Me_4Si. The ultrafiltration fractionation procedure was carried out in a stirred ultrafiltration cell (model 8200, Millipore Corporation, Billerica, MA, USA), fitted with a Biomax-500 ultrafiltration disc (500 000 NMWL).

Plant material

O.sericea was collected on 28.07.2021 in Georgia, nearby city Gori. A voucher specimen (TBPH №22 375) was deposited at the Tbilisi State Medical University I. Kutateladze Institute of Pharmacochemistry.

Extraction and isolation of high-molecular fraction of *O.sericea* roots (HMF-OR)

65.46 g of air-dried and ground *O.sericea* roots was preliminary pretreated in a Soxhlet apparatus with chloroform, methanol and acetone, sequentially, and afforded 57.49 g (87.82 %) roots. Quadruple hot water extraction for 15.5 g of preliminary pretreated of roots afforded 800 ml of mucilage water extract which directly subjected to ultrafiltration and subsequently freeze-dry. The yield of HMF-OR was 0.16 g (0.97 %) based on 17.65 g air-dried biomass.

FTIR of HMF-OR

KBr, υ, cm^{-1}: 3366, 2924.5, 1742, 1610, 1441.5, 1259, 1245.8, 1516.7, 1378, 1022, 1144.6, 1100, 1077, 1050, 9019, 893, 878.4, 854, 831, 786, 759.

RESULTS

HMF-OR was isolated from water mucilage extract of *O.sericea* roots utilizing ultrafiltration using membrane filter with a cut-off value of 500 kDa, as described in some of earlier publications.[2-8,10]

Analyses of HMF-OR was carried out by non-destructive physico-chemical instrumental methods FTIR spectroscopy which is considered as one of the most effective methods to identify the functional groups and nuclear magnetic resonance (NMR) technique which is a powerful tool for structure elucidation of chemical compounds.[16,17]

Study of HMF-OR by FTIR

The FTIR spectroscopy provides se-veral advantages over conventional techniques used in such types of chemical analysis. The FTIR spectrum of HMF-OR sample was interpreted according to the literature data, and the assignments of their infrared absorption bands are reported below.[18-24] The FTIR spectrum of HMF-OR sample shows a broad and intense band centered at 3366 cm^{-1}, which is attributed to the associated OH stretching vibrations. The two bands in the region 2924.5 cm^{-1} are due to aliphatic C–H stretching vibration. The band that appears at 1742 cm^{-1} can be assigned to C=O stretching vibration of methyl esterified carboxylic group. The bands at 1610, 1517, 1441.5, and 1378 cm^{-1} are due to the stretching vibration of C_{ar}=C_{ar} in polar aromatic groups type phenol. The band at 1610 cm^{-1} can be assigned also to the stretching vibration of the free ionic carboxylic group (COO^-). The bands at range of 1259-1246 cm^{-1} correspond to the stretching vibration of C_{ar}-O

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aromatic and/or in plane deformation of CO_2H in carboxylic acids or ethers. The carbohydrates show absorbances between 1441.5 and 1022 cm^{-1} wave numbers values as well, which constitutes the 'fingerprint' region, specific for each polysaccharide. The bands at 1144.5 and 1100 cm^{-1} correspond to R-O-R' (R=R'=Alkyl) ether linkages. The absorption peaks at 1077 and 1050 cm^{-1} were attributed to C–O vibration. The 900-700 cm^{-1} range corresponds to the out of plane deformation in substituted phenolic and polar compounds. The peak near 1078 cm^{-1} indicates that main chemical constituents of HMF-OR might have a pyran ring. The absorption peak at around 1022 cm^{-1} was caused by C-O-C stretching vibration. The appearance of the absorption band near 919 cm^{-1} means that molecules contained β-configuration glycosidic bonds. The absorption peaks at 893, 878.4, and 854 cm^{-1} represent the β-glycosidic bond chain linkage in sugar units, while at 831 cm^{-1} indicated an α-glycosidic bond. Besides, the absorption band at 831 cm^{-1} assigned also to two neighboring hydrogen atoms in aromatic ring. Thus, the FTIR spectrum of HMF-OR showed absorption bands characteristic of phenolcarboxylic acids. Absorption bands corresponding to the hydroxyl groups attached to the aromatic ring, as well as the carboxyl and ether groups were observed. Besides, there were bands characteristic for polysaccharides.

Study of HMF-OR by NMR spectroscopy

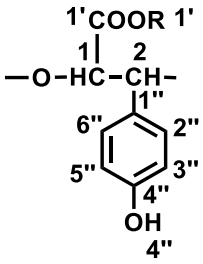
NMR spectroscopy is a powerful technique used to investigate both synthetic and natural compounds in solution and solid state, especially to obtain information at atomic and molecular level by observing the behavior of the atomic nuclei in a magnetic field. Modern pulsed NMR methods are utilized to assign and authenticate low molecular mass structures and provide databases for classical interpretation of polymer spectra. NMR spectroscopy approach has a strong potential to elucidate molecular fragments of compounds contained in complex mixtures.[16,17] Using combined analytical techniques (NMR, FTIR), it is possible to simplify the spectral data and identify a series of principal components that contain information of the sample. In our case study, the interpretation of NMR spectra and obtained data are particularly based on published works. The chemical structures of chemical constituents of HMF-OR sample were identified by comparison of the ^1H NMR spectra with the literature, mainly ^1H NMR chemical shifts.[16-24] Two peaks with chemical shifts 8.9 and 8.1 ppm in ^1H NMR spectrum were assigned to proton of carboxylic groups marked as 1'H and proton for hydroxyl groups of phenol marked as 4''H, respectively (Tab.1, Fig.1). Two signals with chemical shifts of 7.5 and 7.05 ppm obviously belong to couples of aromatic protons marked as 2''H,6''H and 3''H,5''H, respectively (Tab.1, Fig.1). One and the same chemical shifts for pairs of aromatic protons 2''H,6''H (7.5 ppm) and 3''H,5''H (7.05 ppm), respectively, can be explained due to similar localization and surroundings of these pairs, espectively. Consequently, these couples of aromatic protons 2''H,6''H and 3''H,5''H possess similar aromatic properties, respectively. The signal with chemical shift at 5.44 ppm corresponds to proton of oxygen-bound protonated aliphatic carbon atom marked as 1H (Tab.1, Fig.1).

TABLE 1. The signals assignments in the ^1H NMR and ^{13}C NMR spectra of poly[3-(4-hydroxyphenyl)glyceric acid] (PHPGA) from *O.sericea*

Number of H and C atoms	Protons positions	^1H chemical shifts, δ_H, ppm	Positions of carbon atoms	^{13}C chemical shifts, δ_C, ppm
1'	1'R=1'H	8.9	1'COOR	nd*
1'	1'R= -CH_3	3.83	1'R= -CH_3	51.3
1	1H	5.44	1C	72
2	2H	4.76	2C	77
1''	-	-	1''C	130
2'',6''	2''H,6''H	7.5	2''C,6''C	nd*
3'',5''	3''H,5''H	7.05	3''C,5''C	118
4''	4''H	8.1	4''C	143

Abbreviations: nd*, not detected

FIGURE 1. The repeating unit of poly[3-(4-hydroxyphenyl)glyceric acid] (PHPGA); R=H, CH_3.



The corresponding aliphatic carbon atom marked as 1C has δ_C 72 ppm in the ^{13}C NMR spectrum. The signal with chemical shift at 4.76 ppm corresponds to proton of oxygen-bound protonated aliphatic carbon atom marked as 2H. Corresponding aliphatic carbon atom marked as 2C has δ_C 77 ppm in ^{13}C NMR spectrum. The signal with chemical shift at 118 ppm in the ^{13}C NMR spectrum corresponds to a couple of aromatic carbon atoms marked as 3''C and 5''C. One and the same chemical shift for this pair of aromatic carbon atoms 3''H and 5''H can be explained due to similar localization and surroundings of this couple. Unfortunately, we were unable to detect the signals of another couple of aromatic carbon atoms in ^{13}C NMR spectrum marked as 2''C and 6''C and signal of carbon atom of carboxylic group (1'COOR) in ^{13}C NMR spectrum (Tab.1, Fig.1) due to low sensitivity and lack of signal. The signal with chemical shift at 143.66 ppm in ^{13}C NMR spectrum was assigned to aromatic carbon atom marked as 4''C. A resonance in the ^1H NMR spectrum at 3.83 ppm and a signal in the ^{13}C NMR spectrum at 51.3 ppm correspond to O-methyl ester group (-OCH_3) at carboxyl O-methyl ester group (1'$COOCH_3$) (Tab.1, Fig.1). Thus, according to data of FTIR and NMR

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spectroscopies one of the main chemical constituents of HMF-OR was found to be poly[oxy-1-carboxy-2-(4-hydroxyphenyl)ethylene], that is poly[3-(4-hydroxyphenyl)glyceric acid] (PHPGA). Part of carboxyl groups of PHPGA are methylated. However, besides the resonances in NMR spectra belonged to PHPGA, there are signal characteristics for polysaccharides. The chemical shifts assignment was performed by comparison with spectral data already published on the structural characterization of pectins.[25-30] In the ^{1}H NMR spectrum of HMF-OR, the signals at 1.34, 1.64, and 1.78 ppm attributed to the H-6 methyl groups of L-rhamnose of pectin type polysaccharide. The signal at 1.34 ppm corresponds to the units only connected at (1→2) to a galacturonic acid. The signals at 1.64 and 1.78 ppm correspond to the rhamnose units linked by (2→1) bearing a galacturonic acid O-4 branching. The signals at 2.57 and 2.59 ppm were attributed to the acetyl groups attached to D-galacturonic acid residues of pectin type polysaccharide. The former was assigned to 3-O-acetyl groups and the latter to 2-O-acetyl groups linked to O-3 and 2-O of D-galacturonic acid residues, respectively, in the polysaccharide of HMF-OR. A large signal at 3.83 ppm in the ^{1}H NMR spectrum and a resonance in the ^{13}C NMR spectrum at 51.3 ppm was estimated to be the ester linked methyl groups of carboxyl groups in galacturonic acids. The region for anomeric signals contained the following signals at 5.62, 5.60, 5.58, 5.52 and 4.66 ppm for HMF-OR were assigned at least, to α-(1→2)rhamnopyranosyl, α-(1→5)arabinofuranosyl, β-(1→4)galactopyranosyl and α-(1→4)-linked galactopyranuronic acid, respectively. Hydrogen signals at 4.23, 4.3, and 4.66 ppm indicated the presence of β-glycosidic bonds. In the ^{13}C NMR spectrum we noticed the presence in the anomeric regions signals at 99, 98 and 110 ppm characteristics of alternating rhamnose and galacturonic acid units involved in rhamnogalacturonan blocks linked, respectively, (1→2, 1→4), with α-(1→5)-linked arabinofuranose residues in the arabinan side chains. The galactan side-chains were characterized by the minor signals at 105, 77, and 60 ppm assigned to C-1–C-4-C-6, respectively. The signal at 19 ppm was attributed to acetyl group (-OAc) attached to D-galacturonic acid residue. Thus, another main chemical constituent of HMF-OR is complex pectin type polysaccharide.

DISCUSSION

Thus, according to data from FTIR and NMR spectroscopies, the main chemical constituents of HMF-OR were found to be novel *p*-coumaric acid-derived biopolymer PHPGA and complex pectin type polysaccharide.

Pectin is a polysaccharide with a core consisting of α-1,4-linked D-galacturonic acid and α-1,2-L-rhamnose, large number of neutral sugars, including arabinose, galactose, and lesser amounts of other sugars. The structural classification of pectin includes homogalacturonan (HG), rhamnogalacturonan I (RG-I), and substituted galacturonans such as rhamnogalacturonan II (RG-II). According to literature data pectin polysaccharides exhibit various pharmacological activity, such as its immunoregulatory, anti-inflammatory, hypoglycemic, anti-bacterial, antioxidant and antitumor activities. Pectin is employed in several pharmaceutical, cosmetic, food, and biological applications due to its biocompatibility, biodegradability, and non-toxicity.

The data of NMR spectroscopy reveal the presence of altering HG and RG-I in HMF-OR. The RG-I are consisted of a disaccharide repeating unit [→α-D-GalpA-1,2-α-L-Rhap-1,4→] backbone, with side chains contained highly branched α-(1→5)-linked arabinan and short linear β-(1→4)-linked galactan, attached to O-4 of the rhamnnosyl residues. D-galacturonic acid residues might be partially methyl esterified. Thus, HMF-OR has the common characteristics of alterning HG and RG-I blocks, which can be substituted by short galactan and arabinan sidechains. The signals detected in the spectra correspond to the pectins and more specifically to 1,5-arabinan, 1,4-galactan, 1,4-galacturonan and 1,2-rhamnose.

Another main chemical constituent of HMF-OR *p*-coumaric acid-derived biopolymer PHPGA is the regular polymer with a residue of 3-(4-hydroxyphenyl)glyceric acid as the repeating unit. The polyoxyethylene (polyethylene glycol) (PEG) chain is the backbone of PHPGA. Hydroxyphenyl and carboxyl groups are regular substituents at two carbon atoms in the chain. Very often, biopolymers are associated with other biopolymers: polysaccharides, polyphenolics, or proteins, either by covalent bonds or by non-covalent interactions, such as hydrogen bonds, pi bonds, and electrostatic forces. Supramolecular biopolymers are polymeric units derived from macromolecules that can assemble by non-covalent interactions.[31,32]

Hydroxycinnamic acids are phenylpropanoids, derivatives of cinnamic acid, biosynthesized mainly from phenylalanine and tyrosine as secondary metabolites by plants. Among them, *p*-coumaric acid is the most representative. It occurs in plants, both in free and conjugated form, forming derivatives of mono-, oligo- and polysaccharides, organic acids, and amines. *p*-Coumaric acid-derived biopolymer PHPGA is unknown and has been identified for the first time. However synthetic poly(*p*-coumaric acid) was obtained by the thermal polycondensation of the *p*-coumaric acid which showed a photoreaction in the liquid-crystalline state and had excellent cell compatibility.[33] The copolycondensation of *p*-coumaric acid and caffeic acid was performed biodegradable hyperbranched polyester.[34] *p*-Coumaric acid and its derivatives can be found in fruits, vegetables, nuts, grains and mushrooms. *p*-Coumaric acid, caffeic acid and their

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derivatives are known for their diverse physiological functions, including antioxidant, antibacterial, antitumor, anti-inflammatory, and antidiabetic activity, which leads to their use in many fields of industry, such as chemical, food, cosmetics, and pharmaceutical industry.[35]

It is necessary to emphasize that the role of *p*-coumaric acid and its derivatives in plants are not well understood. *p*-Hydroxycinnamic acids, particularly *p*-coumaric acid and its derivatives have received considerable attention because of their intimate association with ability to function as cross-links between polysaccharides and between polysaccharides and the phenylpropanoid polymer.[17]

Unfortunately, we could not quite get the separation of PHPGA and pectic polysaccharide of HMF-OR. On the one hand, this would imply a similar (same order of magnitude) molecular mass for the PHPGA and pectic polysaccharide. On the other hand, due to formation either covalent or hydrogene bonds between PHPGA and the polysaccharide and generation of supramolecular associate. Natural products bearing hydroxyl derivatives of the cinnamic acid moiety have attracted much attention due to their broad spectrum of biological activities and low toxicity. *Trans*-cinnamic acid derivatives have shown to augment the activity of various antibiotics against *Mycobacterium avium* and also show antioxidant, antiinflammatory and cytotoxic properties. Cinnamic acid derivatives bearing phenolic hydroxyl groups also have antioxidant and free radical scavenging properties may produce solid forms with improved pharmaceutical properties.[36]

CONCLUSIONS

PHPGA is a regular polymer with a residue of 3-(4-hydroxyphenyl)glyceric acid as the repeating unit. The polyoxyethylene (polyethylene glycol) (PEG) chain is the backbone of PHPGA. 4-Hydroxyphenyl and carboxyl groups are regular substituents at two carbon atoms in the chain. The complex pectin type polysaccharide has consisted of a disaccharide repeating unit [→α-D-GalpA-1,2-α-L-Rhap-1,4→] backbone, with side chains contained highly branched α-(1→5)-linked arabinan and short linear β-(1→4)-linked galactan, attached to O-4 of the rhamnosyl residues.

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